

COMPOUNDS AND METHODS FOR IMMUNOTHERAPY
AND DIAGNOSIS OF TUBERCULOSIS

Insert **CROSS-REFERENCE TO RELATED APPLICATIONS**

This application is a continuation-in-part of U. S. Application No. 08/730,510, filed October 11, 1996; which claims priority from PCT Application no. PCT/US 96/14674, filed August 30, 1996; and is a continuation-in-part of U.S. Application No. 08/680,574, filed July 12, 1996; which is a continuation-in-part of U.S. Application no. 08/659,683, filed June 5, 1996; which is a continuation-in-part of U.S. Application No. 08/620,874, filed March 22, 1996; which is a continuation-in-part of U.S. Application No. 08/533,634, filed September 22, 1995; which is a continuation-in-part of U.S. Application No. 08/523,436, filed September 1, 1995, now abandoned.

TECHNICAL FIELD

The present invention relates generally to detecting, treating and preventing *Mycobacterium tuberculosis* infection. The invention is more particularly related to polypeptides comprising a *Mycobacterium tuberculosis* antigen, or a portion or other variant thereof, and the use of such polypeptides for diagnosing and vaccinating against *Mycobacterium tuberculosis* infection.

BACKGROUND OF THE INVENTION

Tuberculosis is a chronic, infectious disease, that is generally caused by infection with *Mycobacterium tuberculosis*. It is a major disease in developing countries, as well as an increasing problem in developed areas of the world, with about 8 million new cases and 3 million deaths each year. Although the infection may be asymptomatic for a considerable period of time, the disease is most commonly manifested as an acute inflammation of the lungs, resulting in fever and a nonproductive cough. If left untreated, serious complications and death typically result.

Although tuberculosis can generally be controlled using extended antibiotic therapy, such treatment is not sufficient to prevent the spread of the disease. Infected individuals may be asymptomatic, but contagious, for some time. In addition,

although compliance with the treatment regimen is critical, patient behavior is difficult to monitor. Some patients do not complete the course of treatment, which can lead to ineffective treatment and the development of drug resistance.

Inhibiting the spread of tuberculosis requires effective vaccination and accurate, early diagnosis of the disease. Currently, vaccination with live bacteria is the most efficient method for inducing protective immunity. The most common Mycobacterium employed for this purpose is *Bacillus Calmette-Guerin* (BCG), an avirulent strain of *Mycobacterium bovis*. However, the safety and efficacy of BCG is a source of controversy and some countries, such as the United States, do not vaccinate the general public. Diagnosis is commonly achieved using a skin test, which involves intradermal exposure to tuberculin PPD (protein-purified derivative). Antigen-specific T cell responses result in measurable induration at the injection site by 48-72 hours after injection, which indicates exposure to Mycobacterial antigens. Sensitivity and specificity have, however, been a problem with this test, and individuals vaccinated with BCG cannot be distinguished from infected individuals.

While macrophages have been shown to act as the principal effectors of *M. tuberculosis* immunity, T cells are the predominant inducers of such immunity. The essential role of T cells in protection against *M. tuberculosis* infection is illustrated by the frequent occurrence of *M. tuberculosis* in AIDS patients, due to the depletion of CD4 T cells associated with human immunodeficiency virus (HIV) infection. Mycobacterium-reactive CD4 T cells have been shown to be potent producers of gamma-interferon (IFN- γ), which, in turn, has been shown to trigger the antimycobacterial effects of macrophages in mice. While the role of IFN- γ in humans is less clear, studies have shown that 1,25-dihydroxy-vitamin D3, either alone or in combination with IFN- γ or tumor necrosis factor-alpha, activates human macrophages to inhibit *M. tuberculosis* infection. Furthermore, it is known that IFN- γ stimulates human macrophages to make 1,25-dihydroxy-vitamin D3. Similarly, IL-12 has been shown to play a role in stimulating resistance to *M. tuberculosis* infection. For a review of the immunology of *M. tuberculosis* infection see Chan and Kaufmann in

Tuberculosis: Pathogenesis, Protection and Control, Bloom (ed.), ASM Press, Washington, DC, 1994.

Accordingly, there is a need in the art for improved vaccines and methods for preventing, treating and detecting tuberculosis. The present invention
5 fulfills these needs and further provides other related advantages.

SUMMARY OF THE INVENTION

Briefly stated, this invention provides compounds and methods for preventing and diagnosing tuberculosis. In one aspect, polypeptides are provided
10 comprising an immunogenic portion of a soluble *M. tuberculosis* antigen, or a variant of such an antigen that differs only in conservative substitutions and/or modifications. In one embodiment of this aspect, the soluble antigen has one of the following N-terminal sequences:

- 15 (a) Asp-Pro-Val-Asp-Ala-Val-Ile-Asn-Thr-Thr-Cys-Asn-Tyr-Gly-Gln-Val-Val-Ala-Ala-Leu; (SEQ ID No. 120)
- (b) Ala-Val-Glu-Ser-Gly-Met-Leu-Ala-Leu-Gly-Thr-Pro-Ala-Pro-Ser; (SEQ ID No. 121)
- (c) Ala-Ala-Met-Lys-Pro-Arg-Thr-Gly-Asp-Gly-Pro-Leu-Glu-Ala-Ala-Lys-Glu-Gly-Arg; (SEQ ID No. 122)
- 20 (d) Tyr-Tyr-Trp-Cys-Pro-Gly-Gln-Pro-Phe-Asp-Pro-Ala-Trp-Gly-Pro; (SEQ ID No. 123)
- (e) Asp-Ile-Gly-Ser-Glu-Ser-Thr-Glu-Asp-Gln-Gln-Xaa-Ala-Val; (SEQ ID No. 124)
- (f) Ala-Glu-Glu-Ser-Ile-Ser-Thr-Xaa-Glu-Xaa-Ile-Val-Pro; (SEQ ID
25 No. 125)
- (g) Asp-Pro-Glu-Pro-Ala-Pro-Pro-Val-Pro-Thr-Thr-Ala-Ala-Ser-Pro-Pro-Ser; (SEQ ID No. 126)
- (h) Ala-Pro-Lys-Thr-Tyr-Xaa-Glu-Glu-Leu-Lys-Gly-Thr-Asp-Thr-Gly; (SEQ ID No. 127)

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(c) Y T H A T Y T T A G I V P G I vs-

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sequences, and DNA sequences that hybridize to a sequence recited in SEQ ID Nos.: 26-51, 138 and 139 or a complement thereof under moderately stringent conditions.

In related aspects, DNA sequences encoding the above polypeptides, expression vectors comprising these DNA sequences and host cells transformed or transfected with such expression vectors are also provided.

In another aspect, the present invention provides fusion proteins comprising a first and a second inventive polypeptide or, alternatively, an inventive polypeptide and a known *M. tuberculosis* antigen.

Within other aspects, the present invention provides pharmaceutical compositions that comprise one or more of the above polypeptides, or a DNA molecule encoding such polypeptides, and a physiologically acceptable carrier. The invention also provides vaccines comprising one or more of the polypeptides as described above and a non-specific immune response enhancer, together with vaccines comprising one or more DNA sequences encoding such polypeptides and a non-specific immune response enhancer.

In yet another aspect, methods are provided for inducing protective immunity in a patient, comprising administering to a patient an effective amount of one or more of the above polypeptides.

In further aspects of this invention, methods and diagnostic kits are provided for detecting tuberculosis in a patient. The methods comprise contacting dermal cells of a patient with one or more of the above polypeptides and detecting an immune response on the patient's skin. The diagnostic kits comprise one or more of the above polypeptides in combination with an apparatus sufficient to contact the polypeptide with the dermal cells of a patient.

In yet other aspects, methods are provided for detecting tuberculosis in a patient, such methods comprising contacting dermal cells of a patient with one or more polypeptides encoded by a DNA sequence selected from the group consisting of SEQ ID Nos.: 3, 11, 12, 140 and 141, the complements of said sequences, and DNA sequences that hybridize to a sequence recited in SEQ ID Nos.: 3, 11, 12, 140 and 141;

and detecting an immune response on the patient's skin. Diagnostic kits for use in such methods are also provided.

These and other aspects of the present invention will become apparent upon reference to the following detailed description and attached drawings. All references disclosed herein are hereby incorporated by reference in their entirety as if each was incorporated individually.

BRIEF DESCRIPTION OF THE DRAWINGS AND SEQUENCE IDENTIFIERS

Figure 1A and B illustrate the stimulation of proliferation and interferon- γ production in T cells derived from a first and a second *M. tuberculosis*-immune donor, respectively, by the 14 Kd, 20 Kd and 26 Kd antigens described in Example 1.

Figure 2 illustrates the stimulation of proliferation and interferon- γ production in T cells derived from an *M. tuberculosis*-immune individual by the two representative polypeptides TbRa3 and TbRa9.

Figures 3A-D illustrate the reactivity of antisera raised against secretory *M. tuberculosis* proteins, the known *M. tuberculosis* antigen 85b and the inventive antigens Tb38-1 and TbH-9, respectively, with *M. tuberculosis* lysate (lane 2), *M. tuberculosis* secretory proteins (lane 3), recombinant Tb38-1 (lane 4), recombinant TbH-9 (lane 5) and recombinant 85b (lane 5).

Figure 4A illustrates the stimulation of proliferation in a TbH-9-specific T cell clone by secretory *M. tuberculosis* proteins, recombinant TbH-9 and a control antigen, TbRa11.

Figure 4B illustrates the stimulation of interferon- γ production in a TbH-9-specific T cell clone by secretory *M. tuberculosis* proteins, PPD and recombinant TbH-9.

Figures 5A and B illustrate the stimulation of proliferation and interferon- γ production in TbH9-specific T cells by the fusion protein TbH9-Tb38-1.

Figures 6A and B illustrate the stimulation of proliferation and interferon- γ production in Tb38-1-specific T cells by the fusion protein TbH9-Tb38-1.

SEQ. ID NO. 1 is the DNA sequence of TbRa1.
5 SEQ. ID NO. 2 is the DNA sequence of TbRa10.
SEQ. ID NO. 3 is the DNA sequence of TbRa11.
SEQ. ID NO. 4 is the DNA sequence of TbRa12.
SEQ. ID NO. 5 is the DNA sequence of TbRa13.
SEQ. ID NO. 6 is the DNA sequence of TbRa16.
10 SEQ. ID NO. 7 is the DNA sequence of TbRa17.
SEQ. ID NO. 8 is the DNA sequence of TbRa18.
SEQ. ID NO. 9 is the DNA sequence of TbRa19.
SEQ. ID NO. 10 is the DNA sequence of TbRa24.
SEQ. ID NO. 11 is the DNA sequence of TbRa26.
15 SEQ. ID NO. 12 is the DNA sequence of TbRa28.
SEQ. ID NO. 13 is the DNA sequence of TbRa29.
SEQ. ID NO. 14 is the DNA sequence of TbRa2A.
SEQ. ID NO. 15 is the DNA sequence of TbRa3.
SEQ. ID NO. 16 is the DNA sequence of TbRa32.
20 SEQ. ID NO. 17 is the DNA sequence of TbRa35.
SEQ. ID NO. 18 is the DNA sequence of TbRa36.
SEQ. ID NO. 19 is the DNA sequence of TbRa4.
SEQ. ID NO. 20 is the DNA sequence of TbRa9.
SEQ. ID NO. 21 is the DNA sequence of TbRaB.
25 SEQ. ID NO. 22 is the DNA sequence of TbRaC.
SEQ. ID NO. 23 is the DNA sequence of TbRaD.
SEQ. ID NO. 24 is the DNA sequence of YYWCPG.
SEQ. ID NO. 25 is the DNA sequence of AAMK.
SEQ. ID NO. 26 is the DNA sequence of TbL-23.
30 SEQ. ID NO. 27 is the DNA sequence of TbL-24.

- SEQ. ID NO. 28 is the DNA sequence of TbL-25.
SEQ. ID NO. 29 is the DNA sequence of TbL-28.
SEQ. ID NO. 30 is the DNA sequence of TbL-29.
SEQ. ID NO. 31 is the DNA sequence of TbH-5.
5 SEQ. ID NO. 32 is the DNA sequence of TbH-8.
SEQ. ID NO. 33 is the DNA sequence of TbH-9.
SEQ. ID NO. 34 is the DNA sequence of TbM-1.
SEQ. ID NO. 35 is the DNA sequence of TbM-3.
SEQ. ID NO. 36 is the DNA sequence of TbM-6.
10 SEQ. ID NO. 37 is the DNA sequence of TbM-7.
SEQ. ID NO. 38 is the DNA sequence of TbM-9.
SEQ. ID NO. 39 is the DNA sequence of TbM-12.
SEQ. ID NO. 40 is the DNA sequence of TbM-13.
SEQ. ID NO. 41 is the DNA sequence of TbM-14.
15 SEQ. ID NO. 42 is the DNA sequence of TbM-15.
SEQ. ID NO. 43 is the DNA sequence of TbH-4.
SEQ. ID NO. 44 is the DNA sequence of TbH-4-FWD.
SEQ. ID NO. 45 is the DNA sequence of TbH-12.
SEQ. ID NO. 46 is the DNA sequence of Tb38-1.
20 SEQ. ID NO. 47 is the DNA sequence of Tb38-4.
SEQ. ID NO. 48 is the DNA sequence of TbL-17.
SEQ. ID NO. 49 is the DNA sequence of TbL-20.
SEQ. ID NO. 50 is the DNA sequence of TbL-21.
SEQ. ID NO. 51 is the DNA sequence of TbH-16.
25 SEQ. ID NO. 52 is the DNA sequence of DPEP.
SEQ. ID NO. 53 is the deduced amino acid sequence of DPEP.
SEQ. ID NO. 54 is the protein sequence of DPV N-terminal Antigen.
SEQ. ID NO. 55 is the protein sequence of AVGS N-terminal Antigen.
SEQ. ID NO. 56 is the protein sequence of AAMK N-terminal Antigen.
30 SEQ. ID NO. 57 is the protein sequence of YYWC N-terminal Antigen.

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SEQ. ID NO. 116 is the DNA sequence of Tb38-1F3.

SEQ. ID NO. 117 is the deduced amino acid sequence of Tb38-1F3.

SEQ. ID NO. 115 is the deduced amino acid sequence of Tb38-IN.

SEQ. ID NO. 115 is the deduced amino acid sequence of Tb38-IN.

SEQ. ID NO. 116 is the DNA sequence of Tb38-1F3.

SEQ. ID NO. 117 is the deduced amino acid sequence of Tb38-1F3.

SEQ. ID NO. 114 is the deduced amino acid sequence of Tb37-FL.

SEQ. ID NO. 112 is the DNA sequence of Tb38-1F2 IN.

SEO, ID NO. 111 is the deduced amino acid sequence of TbH-9-4.

SEQ. ID NO. 109 is the deduced amino acid sequence of TbH-9-1.

SEQ ID NO. 108 is the DNA sequence of TbH-9-1.

SEQ. ID NO. 107 is the deduced amino acid sequence

SEO. ID NO. 106 is the DNA sequence of TbH-9FL.

SEQ. ID NO. 104 is the deduced amino acid sequence of ESAT-6.

SEQ. ID NO. 103 is the DNA sequence of ESAT-6.

SEQ. ID NO. 102 is the deduced amino acid sequence of DPV.

SEQ. ID NO. 100 is the deduced amino acid sequence of DPAS.

SEQ. ID NO. 99 is the DNA sequence of DPAS.

SEQ. ID NO. 98 is the amino acid sequence of Tb38-1 Peptide 6.

SEQ. ID NO. 97 is the amino acid sequence of Tb38-1 Peptide 5.

SEQ. ID NO. 96 is the amino acid sequence of Tb38-1 Peptide 4.

SEQ. ID NO. 95 is the amino acid sequence of Tb38-1 Peptide 3.

SEQ. ID NO. 94 is the amino acid sequence of Tb38-1 Peptide 2.

SEQ. ID NO. 93 is the amino acid sequence of Tb38-1 Peptide 1.

SEQ. ID NO. 92 is the deduced amino acid sequence of TbH-12.

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SEQ. ID NO. 125 is the deduced N-terminal amino acid sequence of AEES.

SEQ. ID NO. 126 is the deduced N-terminal amino acid sequence of DPEP.

SEQ. ID NO. 127 is the deduced N-terminal amino acid sequence of APKT.

SEQ. ID NO. 128 is the deduced amino acid sequence of DPAS.

SEQ. ID NO. 129 is the protein sequence of DPPD N-terminal Antigen.

SEQ ID NO. 130-133 are the protein sequences of four DPPD cyanogen bromide fragments.

SEQ ID NO. 134 is the N-terminal protein sequence of XDS antigen.

SEQ ID NO. 135 is the N-terminal protein sequence of AGD antigen.

SEQ ID NO. 136 is the N-terminal protein sequence of APE antigen.

SEQ ID NO. 137 is the N-terminal protein sequence of XYI antigen.

SEQ ID NO. 138 is the DNA sequence of TbH-29.

SEQ ID NO. 139 is the DNA sequence of TbH-30.

SEQ ID NO. 140 is the DNA sequence of TbH-32.

SEQ ID NO. 141 is the DNA sequence of TbH-33.

SEQ ID NO. 142 is the predicted amino acid sequence of TbH-29.

SEQ ID NO. 143 is the predicted amino acid sequence of TbH-30.

SEQ ID NO. 144 is the predicted amino acid sequence of TbH-32.

SEQ ID NO. 145 is the predicted amino acid sequence of TbH-33.

SEQ ID NO: 146-151 are PCR primers used in the preparation of a fusion protein containing TbRa3, 38 kD and Tb38-1.

SEQ ID NO: 152 is the DNA sequence of the fusion protein containing TbRa3, 38 kD and Tb38-1.

SEQ ID NO: 153 is the amino acid sequence of the fusion protein containing TbRa3, 38 kD and Tb38-1.

SEQ ID NO: 154 is the DNA sequence of the *M. tuberculosis* antigen 38 kD.

SEQ ID NO: 155 is the amino acid sequence of the *M. tuberculosis* antigen 38 kD.

DETAILED DESCRIPTION OF THE INVENTION

As noted above, the present invention is generally directed to compositions and methods for preventing, treating and diagnosing tuberculosis. The compositions of the subject invention include polypeptides that comprise at least one immunogenic portion of a *M. tuberculosis* antigen, or a variant of such an antigen that differs only in conservative substitutions and/or modifications. Polypeptides within the scope of the present invention include, but are not limited to, immunogenic soluble *M. tuberculosis* antigens. A "soluble *M. tuberculosis* antigen" is a protein of *M. tuberculosis* origin that is present in *M. tuberculosis* culture filtrate. As used herein, the term "polypeptide" encompasses amino acid chains of any length, including full length proteins (*i.e.*, antigens), wherein the amino acid residues are linked by covalent peptide bonds. Thus, a polypeptide comprising an immunogenic portion of one of the above antigens may consist entirely of the immunogenic portion, or may contain additional sequences. The additional sequences may be derived from the native *M. tuberculosis* antigen or may be heterologous, and such sequences may (but need not) be immunogenic.

"Immunogenic," as used herein, refers to the ability to elicit an immune response (*e.g.*, cellular) in a patient, such as a human, and/or in a biological sample. In particular, antigens that are immunogenic (and immunogenic portions or other variants of such antigens) are capable of stimulating cell proliferation, interleukin-12 production and/or interferon- γ production in biological samples comprising one or more cells selected from the group of T cells, NK cells, B cells and macrophages, where the cells are derived from an *M. tuberculosis*-immune individual. Polypeptides comprising at least an immunogenic portion of one or more *M. tuberculosis* antigens may generally be

The compositions and methods of this invention also encompass variants of the above polypeptides. A "variant," as used herein, is a polypeptide that differs from the native antigen only in conservative substitutions and/or modifications, such that the ability of the polypeptide to induce an immune response is retained. Such variants may generally be identified by modifying one of the above polypeptide sequences, and evaluating the immunogenic properties of the modified polypeptide using, for example, the representative procedures described herein.

20 Variants may also (or alternatively) be modified by, for example, the deletion or addition of amino acids that have minimal influence on the immunogenic properties, secondary structure and hydrophobic nature of the polypeptide. For example, a polypeptide may be conjugated to a signal (or leader) sequence at the N-terminal end of the protein which co-translationally or post-translationally directs transfer of the protein. The polypeptide may also be conjugated to a linker or other sequence for ease of synthesis, purification or identification of the polypeptide (e.g., poly-His), or to enhance binding of the polypeptide to a solid support. For example, a polypeptide may
25 be conjugated to an immunoglobulin Fc region.

In a related aspect, combination polypeptides are disclosed. A "combination polypeptide" is a polypeptide comprising at least one of the above immunogenic portions and one or more additional immunogenic *M. tuberculosis* sequences, which are joined via a peptide linkage into a single amino acid chain. The sequences may be joined directly (*i.e.*, with no intervening amino acids) or may be

joined by way of a linker sequence (e.g., Gly-Cys-Gly) that does not significantly diminish the immunogenic properties of the component polypeptides.

In general, *M. tuberculosis* antigens, and DNA sequences encoding such antigens, may be prepared using any of a variety of procedures. For example, soluble
5 antigens may be isolated from *M. tuberculosis* culture filtrate by procedures known to those of ordinary skill in the art, including anion-exchange and reverse phase chromatography. Purified antigens are then evaluated for their ability to elicit an appropriate immune response (e.g., cellular) using, for example, the representative methods described herein. Immunogenic antigens may then be partially sequenced
10 using techniques such as traditional Edman chemistry. See Edman and Berg, *Eur. J. Biochem.* 80:116-132, 1967.

Immunogenic antigens may also be produced recombinantly using a DNA sequence that encodes the antigen, which has been inserted into an expression vector and expressed in an appropriate host. DNA molecules encoding soluble antigens
15 may be isolated by screening an appropriate *M. tuberculosis* expression library with anti-sera (e.g., rabbit) raised specifically against soluble *M. tuberculosis* antigens. DNA sequences encoding antigens that may or may not be soluble may be identified by screening an appropriate *M. tuberculosis* genomic or cDNA expression library with sera obtained from patients infected with *M. tuberculosis*. Such screens may generally be
20 performed using techniques well known to those of ordinary skill in the art, such as those described in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY, 1989.

DNA sequences encoding soluble antigens may also be obtained by screening an appropriate *M. tuberculosis* cDNA or genomic DNA library for DNA
25 sequences that hybridize to degenerate oligonucleotides derived from partial amino acid sequences of isolated soluble antigens. Degenerate oligonucleotide sequences for use in such a screen may be designed and synthesized, and the screen may be performed, as described (for example) in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY, 1989 (and references cited
30 therein). Polymerase chain reaction (PCR) may also be employed, using the above

oligonucleotides in methods well known in the art, to isolate a nucleic acid probe from a cDNA or genomic library. The library screen may then be performed using the isolated probe.

Alternatively, genomic or cDNA libraries derived from *M. tuberculosis* may be screened directly using peripheral blood mononuclear cells (PBMCs) or T cell lines or clones derived from one or more *M. tuberculosis*-immune individuals. In general, PBMCs and/or T cells for use in such screens may be prepared as described below. Direct library screens may generally be performed by assaying pools of expressed recombinant proteins for the ability to induce proliferation and/or interferon- γ production in T cells derived from an *M. tuberculosis*-immune individual. Alternatively, potential T cell antigens may be first selected based on antibody reactivity, as described above.

Regardless of the method of preparation, the antigens (and immunogenic portions thereof) described herein (which may or may not be soluble) have the ability to induce an immunogenic response. More specifically, the antigens have the ability to induce proliferation and/or cytokine production (*i.e.*, interferon- γ and/or interleukin-12 production) in T cells, NK cells, B cells and/or macrophages derived from an *M. tuberculosis*-immune individual. The selection of cell type for use in evaluating an immunogenic response to a antigen will, of course, depend on the desired response. For example, interleukin-12 production is most readily evaluated using preparations containing B cells and/or macrophages. An *M. tuberculosis*-immune individual is one who is considered to be resistant to the development of tuberculosis by virtue of having mounted an effective T cell response to *M. tuberculosis* (*i.e.*, substantially free of disease symptoms). Such individuals may be identified based on a strongly positive (*i.e.*, greater than about 10 mm diameter induration) intradermal skin test response to tuberculosis proteins (PPD) and an absence of any signs or symptoms of tuberculosis disease. T cells, NK cells, B cells and macrophages derived from *M. tuberculosis*-immune individuals may be prepared using methods known to those of ordinary skill in the art. For example, a preparation of PBMCs (*i.e.*, peripheral blood mononuclear cells) may be employed without further separation of component cells. PBMCs may

generally be prepared, for example, using density centrifugation through Ficoll™ (Winthrop Laboratories, NY). T cells for use in the assays described herein may also be purified directly from PBMCs. Alternatively, an enriched T cell line reactive against mycobacterial proteins, or T cell clones reactive to individual mycobacterial proteins, may be employed. Such T cell clones may be generated by, for example, culturing PBMCs from *M. tuberculosis*-immune individuals with mycobacterial proteins for a period of 2-4 weeks. This allows expansion of only the mycobacterial protein-specific T cells, resulting in a line composed solely of such cells. These cells may then be cloned and tested with individual proteins, using methods known to those of ordinary skill in the art, to more accurately define individual T cell specificity. In general, antigens that test positive in assays for proliferation and/or cytokine production (*i.e.*, interferon- γ and/or interleukin-12 production) performed using T cells, NK cells, B cells and/or macrophages derived from an *M. tuberculosis*-immune individual are considered immunogenic. Such assays may be performed, for example, using the representative procedures described below. Immunogenic portions of such antigens may be identified using similar assays, and may be present within the polypeptides described herein.

The ability of a polypeptide (*e.g.*, an immunogenic antigen, or a portion or other variant thereof) to induce cell proliferation is evaluated by contacting the cells (*e.g.*, T cells and/or NK cells) with the polypeptide and measuring the proliferation of the cells. In general, the amount of polypeptide that is sufficient for evaluation of about 10^5 cells ranges from about 10 ng/mL to about 100 μ g/mL and preferably is about 10 μ g/mL. The incubation of polypeptide with cells is typically performed at 37°C for about six days. Following incubation with polypeptide, the cells are assayed for a proliferative response, which may be evaluated by methods known to those of ordinary skill in the art, such as exposing cells to a pulse of radiolabeled thymidine and measuring the incorporation of label into cellular DNA. In general, a polypeptide that results in at least a three fold increase in proliferation above background (*i.e.*, the proliferation observed for cells cultured without polypeptide) is considered to be able to induce proliferation.

The ability of a polypeptide to stimulate the production of interferon- γ and/or interleukin-12 in cells may be evaluated by contacting the cells with the polypeptide and measuring the level of interferon- γ or interleukin-12 produced by the cells. In general, the amount of polypeptide that is sufficient for the evaluation of about 10^5 cells ranges from about 10 ng/mL to about 100 μ g/mL and preferably is about 10 μ g/mL. The polypeptide may, but need not, be immobilized on a solid support, such as a bead or a biodegradable microsphere, such as those described in U.S. Patent Nos. 4,897,268 and 5,075,109. The incubation of polypeptide with the cells is typically performed at 37°C for about six days. Following incubation with polypeptide, the cells are assayed for interferon- γ and/or interleukin-12 (or one or more subunits thereof), which may be evaluated by methods known to those of ordinary skill in the art, such as an enzyme-linked immunosorbent assay (ELISA) or, in the case of IL-12 P70 subunit, a bioassay such as an assay measuring proliferation of T cells. In general, a polypeptide that results in the production of at least 50 pg of interferon- γ per mL of cultured supernatant (containing 10^4 - 10^5 T cells per mL) is considered able to stimulate the production of interferon- γ . A polypeptide that stimulates the production of at least 10 pg/mL of IL-12 P70 subunit, and/or at least 100 pg/mL of IL-12 P40 subunit, per 10^5 macrophages or B cells (or per 3×10^5 PBMC) is considered able to stimulate the production of IL-12.

In general, immunogenic antigens are those antigens that stimulate proliferation and/or cytokine production (*i.e.*, interferon- γ and/or interleukin-12 production) in T cells, NK cells, B cells and/or macrophages derived from at least about 25% of *M. tuberculosis*-immune individuals. Among these immunogenic antigens, polypeptides having superior therapeutic properties may be distinguished based on the magnitude of the responses in the above assays and based on the percentage of individuals for which a response is observed. In addition, antigens having superior therapeutic properties will not stimulate proliferation and/or cytokine production *in vitro* in cells derived from more than about 25% of individuals that are not *M. tuberculosis*-immune, thereby eliminating responses that are not specifically due to *M. tuberculosis*-responsive cells. Those antigens that induce a response in a high

percentage of T cell, NK cell, B cell and/or macrophage preparations from *M. tuberculosis*-immune individuals (with a low incidence of responses in cell preparations from other individuals) have superior therapeutic properties.

Antigens with superior therapeutic properties may also be identified based on their ability to diminish the severity of *M. tuberculosis* infection in experimental animals, when administered as a vaccine. Suitable vaccine preparations for use on experimental animals are described in detail below. Efficacy may be determined based on the ability of the antigen to provide at least about a 50% reduction in bacterial numbers and/or at least about a 40% decrease in mortality following experimental infection. Suitable experimental animals include mice, guinea pigs and primates.

Antigens having superior diagnostic properties may generally be identified based on the ability to elicit a response in an intradermal skin test performed on an individual with active tuberculosis, but not in a test performed on an individual who is not infected with *M. tuberculosis*. Skin tests may generally be performed as described below, with a response of at least 5 mm induration considered positive.

Immunogenic portions of the antigens described herein may be prepared and identified using well known techniques, such as those summarized in Paul, *Fundamental Immunology*, 3d ed., Raven Press, 1993, pp. 243-247 and references cited therein. Such techniques include screening polypeptide portions of the native antigen for immunogenic properties. The representative proliferation and cytokine production assays described herein may generally be employed in these screens. An immunogenic portion of a polypeptide is a portion that, within such representative assays, generates an immune response (*e.g.*, proliferation, interferon- γ production and/or interleukin-12 production) that is substantially similar to that generated by the full length antigen. In other words, an immunogenic portion of an antigen may generate at least about 20%, and preferably about 100%, of the proliferation induced by the full length antigen in the model proliferation assay described herein. An immunogenic portion may also, or alternatively, stimulate the production of at least about 20%, and preferably about

100%, of the interferon- γ and/or interleukin-12 induced by the full length antigen in the model assay described herein.

Portions and other variants of *M. tuberculosis* antigens may be generated by synthetic or recombinant means. Synthetic polypeptides having fewer than about 100 amino acids, and generally fewer than about 50 amino acids, may be generated using techniques well known to those of ordinary skill in the art. For example, such polypeptides may be synthesized using any of the commercially available solid-phase techniques, such as the Merrifield solid-phase synthesis method, where amino acids are sequentially added to a growing amino acid chain. See Merrifield, *J. Am. Chem. Soc.* 85:2149-2146, 1963. Equipment for automated synthesis of polypeptides is commercially available from suppliers such as Applied BioSystems, Inc., Foster City, CA, and may be operated according to the manufacturer's instructions. Variants of a native antigen may generally be prepared using standard mutagenesis techniques, such as oligonucleotide-directed site-specific mutagenesis. Sections of the DNA sequence may also be removed using standard techniques to permit preparation of truncated polypeptides.

Recombinant polypeptides containing portions and/or variants of a native antigen may be readily prepared from a DNA sequence encoding the polypeptide using a variety of techniques well known to those of ordinary skill in the art. For example, supernatants from suitable host/vector systems which secrete recombinant protein into culture media may be first concentrated using a commercially available filter. Following concentration, the concentrate may be applied to a suitable purification matrix such as an affinity matrix or an ion exchange resin. Finally, one or more reverse phase HPLC steps can be employed to further purify a recombinant protein.

Any of a variety of expression vectors known to those of ordinary skill in the art may be employed to express recombinant polypeptides of this invention. Expression may be achieved in any appropriate host cell that has been transformed or transfected with an expression vector containing a DNA molecule that encodes a recombinant polypeptide. Suitable host cells include prokaryotes, yeast and higher

eukaryotic cells. Preferably, the host cells employed are *E. coli*, yeast or a mammalian cell line such as COS or CHO. The DNA sequences expressed in this manner may encode naturally occurring antigens, portions of naturally occurring antigens, or other variants thereof.

5 In general, regardless of the method of preparation, the polypeptides disclosed herein are prepared in substantially pure form. Preferably, the polypeptides are at least about 80% pure, more preferably at least about 90% pure and most preferably at least about 99% pure. In certain preferred embodiments, described in detail below, the substantially pure polypeptides are incorporated into pharmaceutical
10 compositions or vaccines for use in one or more of the methods disclosed herein.

In certain specific embodiments, the subject invention discloses polypeptides comprising at least an immunogenic portion of a soluble *M. tuberculosis* antigen having one of the following N-terminal sequences, or a variant thereof that differs only in conservative substitutions and/or modifications:

- 15 (a) Asp-Pro-Val-Asp-Ala-Val-Ile-Asn-Thr-Thr-Cys-Asn-Tyr-Gly-Gln-Val-Val-Ala-Ala-Leu; (SEQ ID No. 120)
- (b) Ala-Val-Glu-Ser-Gly-Met-Leu-Ala-Leu-Gly-Thr-Pro-Ala-Pro-Ser; (SEQ ID No. 121)
- (c) Ala-Ala-Met-Lys-Pro-Arg-Thr-Gly-Asp-Gly-Pro-Leu-Glu-Ala-Ala-Lys-Glu-Gly-Arg; (SEQ ID No. 122)
20
- (d) Tyr-Tyr-Trp-Cys-Pro-Gly-Gln-Pro-Phe-Asp-Pro-Ala-Trp-Gly-Pro; (SEQ ID No. 123)
- (e) Asp-Ile-Gly-Ser-Glu-Ser-Thr-Glu-Asp-Gln-Gln-Xaa-Ala-Val; (SEQ ID No. 124)
- 25 (f) Ala-Glu-Glu-Ser-Ile-Ser-Thr-Xaa-Glu-Xaa-Ile-Val-Pro; (SEQ ID No. 125)
- (g) Asp-Pro-Glu-Pro-Ala-Pro-Pro-Val-Pro-Thr-Ala-Ala-Ala-Ser-Pro-Pro-Ser; (SEQ ID No. 126)
- 30 (h) Ala-Pro-Lys-Thr-Tyr-Xaa-Glu-Glu-Leu-Lys-Gly-Thr-Asp-Thr-Gly; (SEQ ID No. 127)

sequences encoded by (a) the DNA sequences of SEQ ID Nos.: 1, 2, 4-10, 13-25 and 52; (b) the complements of such DNA sequences, or (c) DNA sequences substantially homologous to a sequence in (a) or (b).

5 In further specific embodiments, the subject invention discloses polypeptides comprising at least an immunogenic portion of a *M. tuberculosis* antigen (or a variant of such an antigen), which may or may not be soluble, that comprises one or more of the amino acid sequences encoded by (a) the DNA sequences of SEQ ID Nos.: 26-51, 138 and 139, (b) the complements of such DNA sequences or (c) DNA sequences substantially homologous to a sequence in (a) or (b).

10 In the specific embodiments discussed above, the *M. tuberculosis* antigens include variants that are encoded by DNA sequences which are substantially homologous to one or more of DNA sequences specifically recited herein. "Substantial homology," as used herein, refers to DNA sequences that are capable of hybridizing under moderately stringent conditions. Suitable moderately stringent conditions include
15 prewashing in a solution of 5X SSC, 0.5% SDS, 1.0 mM EDTA (pH 8.0); hybridizing at 50°C-65°C, 5X SSC, overnight or, in the case of cross-species homology at 45°C, 0.5X SSC; followed by washing twice at 65°C for 20 minutes with each of 2X, 0.5X and 0.2X SSC containing 0.1% SDS). Such hybridizing DNA sequences are also within the scope of this invention, as are nucleotide sequences that, due to code
20 degeneracy, encode an immunogenic polypeptide that is encoded by a hybridizing DNA sequence.

In a related aspect, the present invention provides fusion proteins comprising a first and a second inventive polypeptide or, alternatively, a polypeptide of the present invention and a known *M. tuberculosis* antigen, such as the 38 kD antigen
25 described in Andersen and Hansen, *Infect. Immun.* 57:2481-2488, 1989, (Genbank Accession No. M30046) or ESAT-6 (SEQ ID Nos. 103 and 104), together with variants of such fusion proteins. The fusion proteins of the present invention may also include a linker peptide between the first and second polypeptides.

A DNA sequence encoding a fusion protein of the present invention is
30 constructed using known recombinant DNA techniques to assemble separate DNA

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sequences encoding the first and second polypeptides into an appropriate expression vector. The 3' end of a DNA sequence encoding the first polypeptide is ligated, with or without a peptide linker, to the 5' end of a DNA sequence encoding the second polypeptide so that the reading frames of the sequences are in phase to permit mRNA translation of the two DNA sequences into a single fusion protein that retains the biological activity of both the first and the second polypeptides.

A peptide linker sequence may be employed to separate the first and the second polypeptides by a distance sufficient to ensure that each polypeptide folds into its secondary and tertiary structures. Such a peptide linker sequence is incorporated into the fusion protein using standard techniques well known in the art. Suitable peptide linker sequences may be chosen based on the following factors: (1) their ability to adopt a flexible extended conformation; (2) their inability to adopt a secondary structure that could interact with functional epitopes on the first and second polypeptides; and (3) the lack of hydrophobic or charged residues that might react with the polypeptide functional epitopes. Preferred peptide linker sequences contain Gly, Asn and Ser residues. Other near neutral amino acids, such as Thr and Ala may also be used in the linker sequence. Amino acid sequences which may be usefully employed as linkers include those disclosed in Maratea et al., *Gene* 40:39-46, 1985; Murphy et al., *Proc. Natl. Acad. Sci. USA* 83:8258-8262, 1986; U.S. Patent No. 4,935,233 and U.S. Patent No. 4,751,180. The linker sequence may be from 1 to about 50 amino acids in length. Peptide sequences are not required when the first and second polypeptides have non-essential N-terminal amino acid regions that can be used to separate the functional domains and prevent steric interference.

The ligated DNA sequences are operably linked to suitable transcriptional or translational regulatory elements. The regulatory elements responsible for expression of DNA are located only 5' to the DNA sequence encoding the first polypeptides. Similarly, stop codons require to end translation and transcription termination signals are only present 3' to the DNA sequence encoding the second polypeptide.

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In another aspect, the present invention provides methods for using one or more of the above polypeptides or fusion proteins (or DNA molecules encoding such polypeptides) to induce protective immunity against tuberculosis in a patient. As used herein, a "patient" refers to any warm-blooded animal, preferably a human. A patient
5 may be afflicted with a disease, or may be free of detectable disease and/or infection. In other words, protective immunity may be induced to prevent or treat tuberculosis.

In this aspect, the polypeptide, fusion protein or DNA molecule is generally present within a pharmaceutical composition and/or a vaccine. Pharmaceutical compositions may comprise one or more polypeptides, each of which
10 may contain one or more of the above sequences (or variants thereof), and a physiologically acceptable carrier. Vaccines may comprise one or more of the above polypeptides and a non-specific immune response enhancer, such as an adjuvant or a liposome (into which the polypeptide is incorporated). Such pharmaceutical compositions and vaccines may also contain other *M. tuberculosis* antigens, either
15 incorporated into a combination polypeptide or present within a separate polypeptide.

Alternatively, a vaccine may contain DNA encoding one or more polypeptides as described above, such that the polypeptide is generated *in situ*. In such vaccines, the DNA may be present within any of a variety of delivery systems known to those of ordinary skill in the art, including nucleic acid expression systems, bacterial
20 and viral expression systems. Appropriate nucleic acid expression systems contain the necessary DNA sequences for expression in the patient (such as a suitable promoter and terminating signal). Bacterial delivery systems involve the administration of a bacterium (such as *Bacillus-Calmette-Guerrin*) that expresses an immunogenic portion of the polypeptide on its cell surface. In a preferred embodiment, the DNA may be
25 introduced using a viral expression system (e.g., vaccinia or other pox virus, retrovirus, or adenovirus), which may involve the use of a non-pathogenic (defective), replication competent virus. Techniques for incorporating DNA into such expression systems are well known to those of ordinary skill in the art. The DNA may also be "naked," as described, for example, in Ulmer et al., *Science* 259:1745-1749, 1993 and reviewed by
30 Cohen, *Science* 259:1691-1692, 1993. The uptake of naked DNA may be increased by

coating the DNA onto biodegradable beads, which are efficiently transported into the cells.

In a related aspect, a DNA vaccine as described above may be administered simultaneously with or sequentially to either a polypeptide of the present invention or a known *M. tuberculosis* antigen, such as the 38 kD antigen described above. For example, administration of DNA encoding a polypeptide of the present invention, either "naked" or in a delivery system as described above, may be followed by administration of an antigen in order to enhance the protective immune effect of the vaccine.

Routes and frequency of administration, as well as dosage, will vary from individual to individual and may parallel those currently being used in immunization using BCG. In general, the pharmaceutical compositions and vaccines may be administered by injection (e.g., intracutaneous, intramuscular, intravenous or subcutaneous), intranasally (e.g., by aspiration) or orally. Between 1 and 3 doses may be administered for a 1-36 week period. Preferably, 3 doses are administered, at intervals of 3-4 months, and booster vaccinations may be given periodically thereafter. Alternate protocols may be appropriate for individual patients. A suitable dose is an amount of polypeptide or DNA that, when administered as described above, is capable of raising an immune response in an immunized patient sufficient to protect the patient from *M. tuberculosis* infection for at least 1-2 years. In general, the amount of polypeptide present in a dose (or produced *in situ* by the DNA in a dose) ranges from about 1 pg to about 100 mg per kg of host, typically from about 10 pg to about 1 mg, and preferably from about 100 pg to about 1 μ g. Suitable dose sizes will vary with the size of the patient, but will typically range from about 0.1 mL to about 5 mL.

While any suitable carrier known to those of ordinary skill in the art may be employed in the pharmaceutical compositions of this invention, the type of carrier will vary depending on the mode of administration. For parenteral administration, such as subcutaneous injection, the carrier preferably comprises water, saline, alcohol, a fat, a wax or a buffer. For oral administration, any of the above carriers or a solid carrier, such as mannitol, lactose, starch, magnesium stearate, sodium saccharine, talcum,

cellulose, glucose, sucrose, and magnesium carbonate, may be employed. Biodegradable microspheres (e.g., polylactic galactide) may also be employed as carriers for the pharmaceutical compositions of this invention. Suitable biodegradable microspheres are disclosed, for example, in U.S. Patent Nos. 4,897,268 and 5,075,109.

5 Any of a variety of adjuvants may be employed in the vaccines of this invention to nonspecifically enhance the immune response. Most adjuvants contain a substance designed to protect the antigen from rapid catabolism, such as aluminum hydroxide or mineral oil, and a nonspecific stimulator of immune responses, such as lipid A, *Bordetella pertussis* or *Mycobacterium tuberculosis*. Suitable adjuvants are
10 commercially available as, for example, Freund's Incomplete Adjuvant and Freund's Complete Adjuvant (Difco Laboratories) and Merck Adjuvant 65 (Merck and Company, Inc., Rahway, NJ). Other suitable adjuvants include alum, biodegradable microspheres, monophosphoryl lipid A and quil A.

In another aspect, this invention provides methods for using one or more
15 of the polypeptides described above to diagnose tuberculosis using a skin test. As used herein, a "skin test" is any assay performed directly on a patient in which a delayed-type hypersensitivity (DTH) reaction (such as swelling, reddening or dermatitis) is measured following intradermal injection of one or more polypeptides as described above. Such injection may be achieved using any suitable device sufficient to contact the
20 polypeptide or polypeptides with dermal cells of the patient, such as a tuberculin syringe or 1 mL syringe. Preferably, the reaction is measured at least 48 hours after injection, more preferably 48-72 hours.

The DTH reaction is a cell-mediated immune response, which is greater in patients that have been exposed previously to the test antigen (i.e., the immunogenic
25 portion of the polypeptide employed, or a variant thereof). The response may be measured visually, using a ruler. In general, a response that is greater than about 0.5 cm in diameter, preferably greater than about 1.0 cm in diameter, is a positive response, indicative of tuberculosis infection, which may or may not be manifested as an active disease.

The polypeptides of this invention are preferably formulated, for use in a skin test, as pharmaceutical compositions containing a polypeptide and a physiologically acceptable carrier, as described above. Such compositions typically contain one or more of the above polypeptides in an amount ranging from about 1 μ g to about 100 μ g, preferably from about 10 μ g to about 50 μ g in a volume of 0.1 mL. Preferably, the carrier employed in such pharmaceutical compositions is a saline solution with appropriate preservatives, such as phenol and/or Tween 80™.

In a preferred embodiment, a polypeptide employed in a skin test is of sufficient size such that it remains at the site of injection for the duration of the reaction period. In general, a polypeptide that is at least 9 amino acids in length is sufficient. The polypeptide is also preferably broken down by macrophages within hours of injection to allow presentation to T-cells. Such polypeptides may contain repeats of one or more of the above sequences and/or other immunogenic or nonimmunogenic sequences.

The following Examples are offered by way of illustration and not by way of limitation.

EXAMPLES

EXAMPLE 1

PURIFICATION AND CHARACTERIZATION OF POLYPEPTIDES FROM *M. TUBERCULOSIS* CULTURE FILTRATE

This example illustrates the preparation of *M. tuberculosis* soluble polypeptides from culture filtrate. Unless otherwise noted, all percentages in the following example are weight per volume.

M. tuberculosis (either H37Ra, ATCC No. 25177, or H37Rv, ATCC No. 25618) was cultured in sterile GAS media at 37°C for fourteen days. The media was then vacuum filtered (leaving the bulk of the cells) through a 0.45 μ filter into a

sterile 2.5 L bottle. The media was next filtered through a 0.2 μ filter into a sterile 4 L bottle and NaN_3 was added to the culture filtrate to a concentration of 0.04%. The bottles were then placed in a 4°C cold room.

-5 The culture filtrate was concentrated by placing the filtrate in a 12 L reservoir that had been autoclaved and feeding the filtrate into a 400 ml Amicon stir cell which had been rinsed with ethanol and contained a 10,000 kDa MWCO membrane. The pressure was maintained at 60 psi using nitrogen gas. This procedure reduced the 12 L volume to approximately 50 ml.

10 The culture filtrate was dialyzed into 0.1% ammonium bicarbonate using a 8,000 kDa MWCO cellulose ester membrane, with two changes of ammonium bicarbonate solution. Protein concentration was then determined by a commercially available BCA assay (Pierce, Rockford, IL).

15 The dialyzed culture filtrate was then lyophilized, and the polypeptides resuspended in distilled water. The polypeptides were dialyzed against 0.01 mM 1,3 bis[tris(hydroxymethyl)-methylamino]propane, pH 7.5 (Bis-Tris propane buffer), the initial conditions for anion exchange chromatography. Fractionation was performed using gel perfusion chromatography on a POROS 146 II Q/M anion exchange column 4.6 mm x 100 mm (Perseptive BioSystems, Framingham, MA) equilibrated in 0.01 mM Bis-Tris propane buffer pH 7.5. Polypeptides were eluted with a linear 0-0.5 M NaCl
20 gradient in the above buffer system. The column eluent was monitored at a wavelength of 220 nm.

The pools of polypeptides eluting from the ion exchange column were dialyzed against distilled water and lyophilized. The resulting material was dissolved in 0.1% trifluoroacetic acid (TFA) pH 1.9 in water, and the polypeptides were purified on
25 a Delta-Pak C18 column (Waters, Milford, MA) 300 Angstrom pore size, 5 micron particle size (3.9 x 150 mm). The polypeptides were eluted from the column with a linear gradient from 0-60% dilution buffer (0.1% TFA in acetonitrile). The flow rate was 0.75 ml/minute and the HPLC eluent was monitored at 214 nm. Fractions containing the eluted polypeptides were collected to maximize the purity of the
30 individual samples. Approximately 200 purified polypeptides were obtained.

The purified polypeptides were then screened for the ability to induce T-cell proliferation in PBMC preparations. The PBMCs from donors known to be PPD skin test positive and whose T-cells were shown to proliferate in response to PPD and crude soluble proteins from MTB were cultured in medium comprising RPMI 1640 supplemented with 10% pooled human serum and 50 $\mu\text{g/ml}$ gentamicin. Purified polypeptides were added in duplicate at concentrations of 0.5 to 10 $\mu\text{g/mL}$. After six days of culture in 96-well round-bottom plates in a volume of 200 μl , 50 μl of medium was removed from each well for determination of IFN- γ levels, as described below. The plates were then pulsed with 1 $\mu\text{Ci/well}$ of tritiated thymidine for a further 18 hours, harvested and tritium uptake determined using a gas scintillation counter. Fractions that resulted in proliferation in both replicates three fold greater than the proliferation observed in cells cultured in medium alone were considered positive.

IFN- γ was measured using an enzyme-linked immunosorbent assay (ELISA). ELISA plates were coated with a mouse monoclonal antibody directed to human IFN- γ (PharMingen, San Diego, CA) in PBS for four hours at room temperature. Wells were then blocked with PBS containing 5% (W/V) non-fat dried milk for 1 hour at room temperature. The plates were then washed six times in PBS/0.2% TWEEN-20 and samples diluted 1:2 in culture medium in the ELISA plates were incubated overnight at room temperature. The plates were again washed and a polyclonal rabbit anti-human IFN- γ serum diluted 1:3000 in PBS/10% normal goat serum was added to each well. The plates were then incubated for two hours at room temperature, washed and horseradish peroxidase-coupled anti-rabbit IgG (Sigma Chemical So., St. Louis, MO) was added at a 1:2000 dilution in PBS/5% non-fat dried milk. After a further two hour incubation at room temperature, the plates were washed and TMB substrate added. The reaction was stopped after 20 min with 1 N sulfuric acid. Optical density was determined at 450 nm using 570 nm as a reference wavelength. Fractions that resulted in both replicates giving an OD two fold greater than the mean OD from cells cultured in medium alone, plus 3 standard deviations, were considered positive.

For sequencing, the polypeptides were individually dried onto Biobrene™ (Perkin Elmer/Applied BioSystems Division, Foster City, CA) treated glass

fiber filters. The filters with polypeptide were loaded onto a Perkin Elmer/Applied BioSystems Division Procise 492 protein sequencer. The polypeptides were sequenced from the amino terminal and using traditional Edman chemistry. The amino acid sequence was determined for each polypeptide by comparing the retention time of the PTH amino acid derivative to the appropriate PTH derivative standards.

Using the procedure described above, antigens having the following N-terminal sequences were isolated:

- (a) Asp-Pro-Val-Asp-Ala-Val-Ile-Asn-Thr-Thr-Xaa-Asn-Tyr-Gly-Gln-Val-Val-Ala-Ala-Leu; (SEQ ID No. 54)
- (b) Ala-Val-Glu-Ser-Gly-Met-Leu-Ala-Leu-Gly-Thr-Pro-Ala-Pro-Ser; (SEQ ID No. 55)
- (c) Ala-Ala-Met-Lys-Pro-Arg-Thr-Gly-Asp-Gly-Pro-Leu-Glu-Ala-Ala-Lys-Glu-Gly-Arg; (SEQ ID No. 56)
- (d) Tyr-Tyr-Trp-Cys-Pro-Gly-Gln-Pro-Phe-Asp-Pro-Ala-Trp-Gly-Pro; (SEQ ID No. 57)
- (e) Asp-Ile-Gly-Ser-Glu-Ser-Thr-Glu-Asp-Gln-Gln-Xaa-Ala-Val; (SEQ ID No. 58)
- (f) Ala-Glu-Glu-Ser-Ile-Ser-Thr-Xaa-Glu-Xaa-Ile-Val-Pro; (SEQ ID No. 59)
- (g) Asp-Pro-Glu-Pro-Ala-Pro-Pro-Val-Pro-Thr-Ala-Ala-Ala-Ala-Pro-Pro-Ala; (SEQ ID No. 60) and
- (h) Ala-Pro-Lys-Thr-Tyr-Xaa-Glu-Glu-Leu-Lys-Gly-Thr-Asp-Thr-Gly; (SEQ ID No. 61)

wherein Xaa may be any amino acid.

An additional antigen was isolated employing a microbore HPLC purification step in addition to the procedure described above. Specifically, 20 μ l of a fraction comprising a mixture of antigens from the chromatographic purification step previously described, was purified on an Aquapore C18 column (Perkin Elmer/Applied Biosystems Division, Foster City, CA) with a 7 micron pore size, column size 1 mm x 100 mm, in a Perkin Elmer/Applied Biosystems Division Model 172 HPLC. Fractions

were eluted from the column with a linear gradient of 1%/minute of acetonitrile (containing 0.05% TFA) in water (0.05% TFA) at a flow rate of 80 μ l/minute. The eluent was monitored at 250 nm. The original fraction was separated into 4 major peaks plus other smaller components and a polypeptide was obtained which was shown to have a molecular weight of 12.054 Kd (by mass spectrometry) and the following N-terminal sequence:

(i) Asp-Pro-Ala-Ser-Ala-Pro-Asp-Val-Pro-Thr-Ala-Ala-Gln-Gln-Thr-Ser-Leu-Leu-Asn-Asn-Leu-Ala-Asp-Pro-Asp-Val-Ser-Phe-Ala-Asp (SEQ ID No. 62).

10 This polypeptide was shown to induce proliferation and IFN- γ production in PBMC preparations using the assays described above.

Additional soluble antigens were isolated from *M. tuberculosis* culture filtrate as follows. *M. tuberculosis* culture filtrate was prepared as described above. Following dialysis against Bis-Tris propane buffer, at pH 5.5, fractionation was performed using anion exchange chromatography on a Poros QE column 4.6 x 100 mm (Perseptive Biosystems) equilibrated in Bis-Tris propane buffer pH 5.5. Polypeptides were eluted with a linear 0-1.5 M NaCl gradient in the above buffer system at a flow rate of 10 ml/min. The column eluent was monitored at a wavelength of 214 nm.

The fractions eluting from the ion exchange column were pooled and subjected to reverse phase chromatography using a Poros R2 column 4.6 x 100 mm (Perseptive Biosystems). Polypeptides were eluted from the column with a linear gradient from 0-100% acetonitrile (0.1% TFA) at a flow rate of 5 ml/min. The eluent was monitored at 214 nm.

Fractions containing the eluted polypeptides were lyophilized and resuspended in 80 μ l of aqueous 0.1% TFA and further subjected to reverse phase chromatography on a Vydac C4 column 4.6 x 150 mm (Western Analytical, Temecula, CA) with a linear gradient of 0-100% acetonitrile (0.1% TFA) at a flow rate of 2 ml/min. Eluent was monitored at 214 nm.

The fraction with biological activity was separated into one major peak plus other smaller components. Western blot of this peak onto PVDF membrane

revealed three major bands of molecular weights 14 Kd, 20 Kd and 26 Kd. These polypeptides were determined to have the following N-terminal sequences, respectively:

- (j) Xaa-Asp-Ser-Glu-Lys-Ser-Ala-Thr-Ile-Lys-Val-Thr-Asp-Ala-Ser; (SEQ ID No. 134)
- 5 (k) Ala-Gly-Asp-Thr-Xaa-Ile-Tyr-Ile-Val-Gly-Asn-Leu-Thr-Ala-Asp; (SEQ ID No. 135) and
- (l) Ala-Pro-Glu-Ser-Gly-Ala-Gly-Leu-Gly-Gly-Thr-Val-Gln-Ala-Gly; (SEQ ID No. 136), wherein Xaa may be any amino acid.

Using the assays described above, these polypeptides were shown to induce proliferation and IFN- γ production in PBMC preparations. Figs. 1A and B show the results of such assays using PBMC preparations from a first and a second donor, respectively.

DNA sequences that encode the antigens designated as (a), (c), (d) and (g) above were obtained by screening a genomic *M. tuberculosis* library using ^{32}P end labeled degenerate oligonucleotides corresponding to the N-terminal sequence and containing *M. tuberculosis* codon bias. The screen performed using a probe corresponding to antigen (a) above identified a clone having the sequence provided in SEQ ID No. 101. The polypeptide encoded by SEQ ID No. 101 is provided in SEQ ID No. 102. The screen performed using a probe corresponding to antigen (g) above identified a clone having the sequence provided in SEQ ID No. 52. The polypeptide encoded by SEQ ID No. 52 is provided in SEQ ID No. 53. The screen performed using a probe corresponding to antigen (d) above identified a clone having the sequence provided in SEQ ID No. 24, and the screen performed with a probe corresponding to antigen (c) identified a clone having the sequence provided in SEQ ID No: 25.

25 The above amino acid sequences were compared to known amino acid sequences in the gene bank using the DNA STAR system. The database searched contains some 173,000 proteins and is a combination of the Swiss, PIR databases along with translated protein sequences (Version 87). No significant homologies to the amino acid sequences for antigens (a)-(h) and (l) were detected.

The amino acid sequence for antigen (i) was found to be homologous to a sequence from *M. leprae*. The full length *M. leprae* sequence was amplified from genomic DNA using the sequence obtained from GENBANK. This sequence was then used to screen the *M. tuberculosis* library described below in Example 2 and a full length copy of the *M. tuberculosis* homologue was obtained (SEQ ID No. 99).

The amino acid sequence for antigen (j) was found to be homologous to a known *M. tuberculosis* protein translated from a DNA sequence. To the best of the inventors' knowledge, this protein has not been previously shown to possess T-cell stimulatory activity. The amino acid sequence for antigen (k) was found to be related to a sequence from *M. leprae*.

In the proliferation and IFN- γ assays described above, using three PPD positive donors, the results for representative antigens provided above are presented in Table 1:

TABLE 1
RESULTS OF PBMC PROLIFERATION AND IFN- γ ASSAYS

Sequence	Proliferation	IFN- γ
(a)	+	-
(c)	+++	+++
(d)	++	++
(g)	+++	+++
(h)	+++	+++

In Table 1, responses that gave a stimulation index (SI) of between 2 and 4 (compared to cells cultured in medium alone) were scored as +, an SI of 4-8 or 2-4 at a concentration of 1 μ g or less was scored as ++ and an SI of greater than 8 was scored as +++. The antigen of sequence (i) was found to have a high SI (+++) for one donor and lower SI (++ and +) for the two other donors in both proliferation and IFN- γ assays.

These results indicate that these antigens are capable of inducing proliferation and/or interferon- γ production.

EXAMPLE 2

5 USE OF PATIENT SERA TO ISOLATE *M. TUBERCULOSIS* ANTIGENS

This example illustrates the isolation of antigens from *M. tuberculosis* lysate by screening with serum from *M. tuberculosis*-infected individuals.

Dessicated *M. tuberculosis* H37Ra (Difco Laboratories) was added to a
 10 2% NP40 solution, and alternately homogenized and sonicated three times. The resulting suspension was centrifuged at 13,000 rpm in microfuge tubes and the supernatant put through a 0.2 micron syringe filter. The filtrate was bound to Macro Prep DEAE beads (BioRad, Hercules, CA). The beads were extensively washed with 20 mM Tris pH 7.5 and bound proteins eluted with 1M NaCl. The 1M NaCl elute was
 15 dialyzed overnight against 10 mM Tris, pH 7.5. Dialyzed solution was treated with DNase and RNase at 0.05 mg/ml for 30 min. at room temperature and then with α -D-mannosidase, 0.5 U/mg at pH 4.5 for 3-4 hours at room temperature. After returning to pH 7.5, the material was fractionated via FPLC over a Bio Scale-Q-20 column (BioRad). Fractions were combined into nine pools, concentrated in a Centriprep 10
 20 (Amicon, Beverley, MA) and then screened by Western blot for serological activity using a serum pool from *M. tuberculosis*-infected patients which was not immunoreactive with other antigens of the present invention.

The most reactive fraction was run in SDS-PAGE and transferred to PVDF. A band at approximately 85 Kd was cut out yielding the sequence:

25 (m) Xaa-Tyr-Ile-Ala-Tyr-Xaa-Thr-Thr-Ala-Gly-Ile-Val-Pro-Gly-Lys-Ile-Asn-Val-His-Leu-Val; (SEQ ID No. 137), wherein Xaa may be any amino acid.

Comparison of this sequence with those in the gene bank as described above, revealed no significant homologies to known sequences.

EXAMPLE 3

PREPARATION OF DNA SEQUENCES ENCODING *M. TUBERCULOSIS* ANTIGENS

This example illustrates the preparation of DNA sequences encoding
5 *M. tuberculosis* antigens by screening a *M. tuberculosis* expression library with sera
obtained from patients infected with *M. tuberculosis*, or with anti-sera raised against
soluble *M. tuberculosis* antigens.

10 A. PREPARATION OF *M. TUBERCULOSIS* SOLUBLE ANTIGENS USING RABBIT ANTI-SERA

Genomic DNA was isolated from the *M. tuberculosis* strain H37Ra. The
DNA was randomly sheared and used to construct an expression library using the
Lambda ZAP expression system (Stratagene, La Jolla, CA). Rabbit anti-sera was
generated against secretory proteins of the *M. tuberculosis* strains H37Ra, H37Rv and
15 Erdman by immunizing a rabbit with concentrated supernatant of the *M. tuberculosis*
cultures. Specifically, the rabbit was first immunized subcutaneously with 200 µg of
protein antigen in a total volume of 2 ml containing 10 µg muramyl dipeptide
(Calbiochem, La Jolla, CA) and 1 ml of incomplete Freund's adjuvant. Four weeks later
the rabbit was boosted subcutaneously with 100 µg antigen in incomplete Freund's
20 adjuvant. Finally, the rabbit was immunized intravenously four weeks later with 50 µg
protein antigen. The anti-sera were used to screen the expression library as described in
Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor
Laboratories, Cold Spring Harbor, NY, 1989. Bacteriophage plaques expressing
immunoreactive antigens were purified. Phagemid from the plaques was rescued and
25 the nucleotide sequences of the *M. tuberculosis* clones deduced.

Thirty two clones were purified. Of these, 25 represent sequences that
have not been previously identified in human *M. tuberculosis*. Recombinant antigens
were expressed and purified antigens used in the Immunological analysis described in
Example 1. Proteins were induced by IPTG and purified by gel elution, as described in
30 Skeiky et al., *J. Exp. Med.* 181:1527-1537, 1995. Representative sequences of DNA

molecules identified in this screen are provided in SEQ ID Nos.: 1-25. The corresponding predicted amino acid sequences are shown in SEQ ID Nos. 63-87.

On comparison of these sequences with known sequences in the gene bank using the databases described above, it was found that the clones referred to hereinafter as TbRA2A, TbRA16, TbRA18, and TbRA29 (SEQ ID Nos. 76, 68, 70, 75) show some homology to sequences previously identified in *Mycobacterium leprae* but not in *M. tuberculosis*. TbRA11, TbRA26, TbRA28 and TbDPEP (SEQ ID Nos.: 65, 73, 74, 53) have been previously identified in *M. tuberculosis*. No significant homologies were found to TbRA1, TbRA3, TbRA4, TbRA9, TbRA10, TbRA13, TbRA17, TbRa19, TbRA29, TbRA32, TbRA36 and the overlapping clones TbRA35 and TbRA12 (SEQ ID Nos. 63, 77, 81, 82, 64, 67, 69, 71, 75, 78, 80, 79, 66). The clone TbRa24 is overlapping with clone TbRa29.

The results of PBMC proliferation and interferon- γ assays performed on representative recombinant antigens, and using T-cell preparations from several different *M. tuberculosis*-immune patients, are presented in Tables 2 and 3, respectively.

TABLE 2
RESULTS OF PBMC PROLIFERATION TO REPRESENTATIVE SOLUBLE ANTIGENS

Antigen	Patient												
	1	2	3	4	5	6	7	8	9	10	11	12	13
TbRa1	-	-	±	++	-	±	±	±	-	-	+	±	-
TbRa3	-	±	++	-	±	-	-	++	±	-	-	-	-
TbRa9	-	-	nt	nt	++	++	nt	nt	nt	nt	nt	nt	nt
TbRa10	-	-	±	±	±	+	nt	±	-	+	±	±	-
TbRa11	±	±	+	++	++	+	nt	-	++	++	++	±	nt
TbRa12	-	-	+	+	±	++	+	±	±	-	+	-	-
TbRa16	nt	nt	nt	nt	-	+	nt	nt	nt	nt	nt	nt	nt
TbRa24	nt	nt	nt	nt	-	-	nt	nt	nt	nt	nt	nt	nt
TbRa26	-	+	nt	nt	-	-	nt	nt	nt	nt	nt	nt	nt
TbRa29	nt	nt	nt	nt	-	-	nt	nt	nt	nt	nt	nt	nt
TbRa35	++	nt	++	++	++	++	nt	++	++	++	++	++	nt
TbRaB	nt	nt	nt	nt	-	-	nt	nt	nt	nt	nt	nt	nt
TbRaC	nt	nt	nt	nt	-	-	nt	nt	nt	nt	nt	nt	nt
TbRaD	nt	nt	nt	nt	-	-	nt	nt	nt	nt	nt	nt	nt
AAMK	-	-	±	-	-	-	nt	-	-	-	nt	±	nt
YY	-	-	-	-	-	-	nt	-	-	-	nt	+	nt
DPEP	-	+	-	++	-	-	nt	++	±	+	±	±	nt
Control	-	-	-	-	-	-	-	-	-	-	-	-	-

nt = not tested

TABLE 3

[illegible]

In Tables 2 and 3, responses that gave a stimulation index (SI) of between 1.2 and 2 (compared to cells cultured in medium alone) were scored as \pm , a SI of 2-4 was scored as +, as SI of 4-8 or 2-4 at a concentration of 1 μ g or less was scored as ++ and an SI of greater than 8 was scored as +++. In addition, the effect of concentration on proliferation and interferon- γ production is shown for two of the above antigens in the attached Figure. For both proliferation and interferon- γ production, TbRa3 was scored as ++ and TbRa9 as +.

These results indicate that these soluble antigens can induce proliferation and/or interferon- γ production in T-cells derived from an *M. tuberculosis*-immune individual.

B. USE OF PATIENT SERA TO IDENTIFY DNA SEQUENCES ENCODING
M. TUBERCULOSIS ANTIGENS

The genomic DNA library described above, and an additional H37Rv library, were screened using pools of sera obtained from patients with active tuberculosis. To prepare the H37Rv library, *M. tuberculosis* strain H37Rv genomic DNA was isolated, subjected to partial *Sau*3A digestion and used to construct an expression library using the Lambda Zap expression system (Stratagene, La Jolla, Ca). Three different pools of sera, each containing sera obtained from three individuals with active pulmonary or pleural disease, were used in the expression screening. The pools were designated TbL, TbM and TbH, referring to relative reactivity with H37Ra lysate (*i.e.*, TbL = low reactivity, TbM = medium reactivity and TbH = high reactivity) in both ELISA and immunoblot format. A fourth pool of sera from seven patients with active pulmonary tuberculosis was also employed. All of the sera lacked increased reactivity with the recombinant 38 kD *M. tuberculosis* H37Ra phosphate-binding protein.

All pools were pre-adsorbed with *E. coli* lysate and used to screen the H37Ra and H37Rv expression libraries, as described in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY, 1989. Bacteriophage plaques expressing immunoreactive antigens were purified. Phagemid from the plaques was rescued and the nucleotide sequences of the *M. tuberculosis* clones deduced.

Thirty two clones were purified. Of these, 31 represented sequences that had not been previously identified in human *M. tuberculosis*. Representative sequences of the DNA molecules identified are provided in SEQ ID Nos.: 26-51 and 105. Of these, TbH-8-2 (SEQ. ID NO. 105) is a partial clone of TbH-8, and TbH-4 (SEQ. ID NO. 43) and TbH-4-FWD (SEQ. ID NO. 44) are non-contiguous sequences from the same clone. Amino acid sequences for the antigens hereinafter identified as Tb38-1, TbH-4, TbH-8, TbH-9, and TbH-12 are shown in SEQ ID Nos.: 88-92. Comparison of these sequences with known sequences in the gene bank using the databases identified above revealed no significant homologies to TbH-4, TbH-8, TbH-9 and TbM-3, although weak homologies were found to TbH-9. TbH-12 was found to be homologous to a 34 kD antigenic protein previously identified in *M. paratuberculosis* (Acc. No. S28515). Tb38-1 was found to be located 34 base pairs upstream of the open reading frame for the antigen ESAT-6 previously identified in *M. bovis* (Acc. No. U34848) and in *M. tuberculosis* (Sorensen et al., *Infect. Immun.* 63:1710-1717, 1995).

Probes derived from Tb38-1 and TbH-9, both isolated from an H37Ra library, were used to identify clones in an H37Rv library. Tb38-1 hybridized to Tb38-1F2, Tb38-1F3, Tb38-1F5 and Tb38-1F6 (SEQ. ID NOS. 112, 113, 116, 118, and 119). (SEQ ID NOS. 112 and 113 are non-contiguous sequences from clone Tb38-1F2.) Two open reading frames were deduced in Tb38-1F2; one corresponds to Tb37FL (SEQ. ID. NO. 114), the second, a partial sequence, may be the homologue of Tb38-1 and is called Tb38-IN (SEQ. ID NO. 115). The deduced amino acid sequence of Tb38-1F3 is presented in SEQ. ID. NO. 117. A TbH-9 probe identified three clones in the H37Rv library: TbH-9-FL (SEQ. ID NO. 106), which may be the homologue of TbH-9 (H37Ra), TbH-9-1 (SEQ. ID NO. 108), and TbH-9-4 (SEQ. ID NO. 110), all of which are highly related sequences to TbH-9. The deduced amino acid sequences for these three clones are presented in SEQ ID NOS. 107, 109 and 111.

Further screening of the *M. tuberculosis* genomic DNA library, as described above, resulted in the recovery of ten additional reactive clones, representing seven different genes. One of these genes was identified as the 38 Kd antigen discussed

above, one was determined to be identical to the 14Kd alpha crystallin heat shock protein previously shown to be present in *M. tuberculosis*, and a third was determined to be identical to the antigen TbH-8 described above. The determined DNA sequences for the remaining five clones (hereinafter referred to as TbH-29, TbH-30, TbH-32 and
5 TbH-33) are provided in SEQ ID NO: 138-141, respectively, with the corresponding predicted amino acid sequences being provided in SEQ ID NO: 142-145, respectively. The DNA and amino acid sequences for these antigens were compared with those in the gene bank as described above. No homologies were found to the 5' end of TbH-29 (which contains the reactive open reading frame), although the 3' end of TbH-29 was
10 found to be identical to the *M. tuberculosis* cosmid Y227. TbH-32 and TbH-33 were found to be identical to the previously identified *M. tuberculosis* insertion element IS6110 and to the *M. tuberculosis* cosmid Y50, respectively. No significant homologies to TbH-30 were found.

Positive phagemid from this additional screening were used to infect *E.*
15 *coli* XL-1 Blue MRF', as described in Sambrook et al., *supra*. Induction of recombinant protein was accomplished by the addition of IPTG. Induced and uninduced lysates were run in duplicate on SDS-PAGE and transferred to nitrocellulose filters. Filters were reacted with human *M. tuberculosis* sera (1:200 dilution) reactive with TbH and a rabbit sera (1:200 or 1:250 dilution) reactive with the N-terminal 4 Kd portion of lacZ.
20 Sera incubations were performed for 2 hours at room temperature. Bound antibody was detected by addition of ¹²⁵I-labeled Protein A and subsequent exposure to film for variable times ranging from 16 hours to 11 days. The results of the immunoblots are summarized in Table 4.

TABLE 4

<u>Antigen</u>	<u>Human M. tb Sera</u>	<u>Anti-lacZ Sera</u>
TbH-29	45 Kd	45 Kd
TbH-30	No reactivity	29 Kd
TbH-32	12 Kd	12 Kd
TbH-33	16 Kd	16 Kd

Positive reaction of the recombinant human *M. tuberculosis* antigens with both the human *M. tuberculosis* sera and anti-lacZ sera indicate that reactivity of the human *M. tuberculosis* sera is directed towards the fusion protein. Antigens reactive with the anti-lacZ sera but not with the human *M. tuberculosis* sera may be the result of the human *M. tuberculosis* sera recognizing conformational epitopes, or the antigen-antibody binding kinetics may be such that the 2 hour sera exposure in the immunoblot is not sufficient.

The results of T-cell assays performed on Tb38-1, ESAT-6 and other representative recombinant antigens are presented in Tables 5A, B and 6, respectively, below:

TABLE 5A
RESULTS OF PBMC PROLIFERATION TO REPRESENTATIVE ANTIGENS

Antigen	Donor										
	1	2	3	4	5	6	7	8	9	10	11
Tb38.1	+++	+	-	-	-	++	-	+	-	++	+++
ESAT-6	+++	+	+	+	-	+	-	+	+	++	+++
TbH-9	++	++	-	++	±	±	++	++	++	++	++

TABLE 5B
RESULTS OF PBMC INTERFERON- γ PRODUCTION TO REPRESENTATIVE ANTIGENS

Antigen	Donor										
	1	2	3	4	5	6	7	8	9	10	11
Tb38.1	+++	+	-	+	+	+++	-	++	-	+++	+++
ESAT-6	+++	+	+	+	+-	+	-	+	+	+++	+++
TbH-9	++	++	-	+++	\pm	\pm	+++	+++	++	+++	++

5

TABLE 6
SUMMARY OF T-CELL RESPONSES TO REPRESENTATIVE ANTIGENS

Antigen	Proliferation			Interferon- γ			total
	patient 4	patient 5	patient 6	patient 4	patient 5	patient 6	
TbH9	++	++	++	+++	++	++	13
TbM7	-	+	-	++	+	-	4
TbH5	-	+	+	++	++	++	8
TbL23	-	+	\pm	++	++	+	7.5
TbH4	-	++	\pm	++	++	\pm	7
- control	-	-	-	-	-	-	0

10

These results indicate that both the inventive *M. tuberculosis* antigens and ESAT-6 can induce proliferation and/or interferon- γ production in T-cells derived from an *M. tuberculosis*-immune individual. To the best of the inventors' knowledge, ESAT-6 has not been previously shown to stimulate human immune responses

A set of six overlapping peptides covering the amino acid sequence of the antigen Tb38-1 was constructed using the method described in Example 6. The sequences of these peptides, hereinafter referred to as pep1-6, are provided in SEQ ID Nos. 93-98, respectively. The results of T-cell assays using these peptides are shown in Tables 7 and 8. These results confirm the existence, and help to localize T-cell epitopes within Tb38-1 capable of inducing proliferation and interferon- γ production in T-cells derived from an *M. tuberculosis* immune individual.

20

TABLE 7
RESULTS OF PBMC PROLIFERATION TO TB38-1 PEPTIDES

[illegible]

[illegible][illegible]

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IFN- γ by a second TbH-9-specific T cell clone (designated PPD 800-10) prepared from PBMC from a healthy PPD-positive donor, following stimulation of the T cell clone with secretory proteins, PPD or recombinant TbH-9. These results further confirm that TbH-9 is not secreted by *M. tuberculosis*.

EXAMPLE 4

PURIFICATION AND CHARACTERIZATION OF A POLYPEPTIDE FROM TUBERCULIN PURIFIED PROTEIN DERIVATIVE

An *M. tuberculosis* polypeptide was isolated from tuberculin purified protein derivative (PPD) as follows.

PPD was prepared as published with some modification (Seibert, F. et al., Tuberculin purified protein derivative. Preparation and analyses of a large quantity for standard. The American Review of Tuberculosis 44:9-25, 1941).

M. tuberculosis Rv strain was grown for 6 weeks in synthetic medium in roller bottles at 37°C. Bottles containing the bacterial growth were then heated to 100°C in water vapor for 3 hours. Cultures were sterile filtered using a 0.22 μ filter and the liquid phase was concentrated 20 times using a 3 kD cut-off membrane. Proteins were precipitated once with 50% ammonium sulfate solution and eight times with 25% ammonium sulfate solution. The resulting proteins (PPD) were fractionated by reverse phase liquid chromatography (RP-HPLC) using a C18 column (7.8 x 300 mM; Waters, Milford, MA) in a Biocad HPLC system (Perseptive Biosystems, Framingham, MA). Fractions were eluted from the column with a linear gradient from 0-100% buffer (0.1% TFA in acetonitrile). The flow rate was 10 ml/minute and eluent was monitored at 214 nm and 280 nm.

Six fractions were collected, dried, suspended in PBS and tested individually in *M. tuberculosis*-infected guinea pigs for induction of delayed type hypersensitivity (DTH) reaction. One fraction was found to induce a strong DTH reaction and was subsequently fractionated further by RP-HPLC on a microbore Vydac

C18 column (Cat. No. 218TP5115) in a Perkin Elmer/Applied Biosystems Division Model 172 HPLC. Fractions were eluted with a linear gradient from 5-100% buffer (0.05% TFA in acetonitrile) with a flow rate of 80 μ l/minute. Eluent was monitored at 215 nm. Eight fractions were collected and tested for induction of DTH in *M. tuberculosis*-infected guinea pigs. One fraction was found to induce strong DTH of about 16 mm induration. The other fractions did not induce detectable DTH. The positive fraction was submitted to SDS-PAGE gel electrophoresis and found to contain a single protein band of approximately 12 kD molecular weight.

This polypeptide, herein after referred to as DPPD, was sequenced from the amino terminal using a Perkin Elmer/Applied Biosystems Division Procise 492 protein sequencer as described above and found to have the N-terminal sequence shown in SEQ ID No.: 129. Comparison of this sequence with known sequences in the gene bank as described above revealed no known homologies. Four cyanogen bromide fragments of DPPD were isolated and found to have the sequences shown in SEQ ID Nos.: 130-133.

The ability of the antigen DPPD to stimulate human PBMC to proliferate and to produce IFN- γ was assayed as described in Example 1. As shown in Table 9, DPPD was found to stimulate proliferation and elicit production of large quantities of IFN- γ ; more than that elicited by commercial PPD.

TABLE 9
RESULTS OF PROLIFERATION AND INTERFERON- γ ASSAYS TO DPPD

PBMC Donor	Stimulator	Proliferation (CPM)	IFN- γ (OD ₄₅₀)
A	Medium	1,089	0.17
	PPD (commercial)	8,394	1.29
	DPPD	13,451	2.21
B	Medium	450	0.09
	PPD (commercial)	3,929	1.26
	DPPD	6,184	1.49
C	Medium	541	0.11
	PPD (commercial)	8,907	0.76
	DPPD	23,024	>2.70

5

EXAMPLE 5

USE OF REPRESENTATIVE ANTIGENS FOR DIAGNOSIS OF TUBERCULOSIS

This example illustrates the effectiveness of several representative polypeptides in skin tests for the diagnosis of *M. tuberculosis* infection.

Individuals were injected intradermally with 100 μ l of either PBS or PBS plus Tween 20TM containing either 0.1 μ g of protein (for TbH-9 and TbRa35) or 1.0 μ g of protein (for TbRa38-1). Induration was measured between 5-7 days after injection, with a response of 5 mm or greater being considered positive. Of the 20 individuals tested, 2 were PPD negative and 18 were PPD positive. Of the PPD positive individuals, 3 had active tuberculosis, 3 had been previously infected with tuberculosis and 9 were healthy. In a second study, 13 PPD positive individuals were tested with 0.1 μ g TbRa11 in either PBS or PBS plus Tween 20TM as described above. The results of both studies are shown in Table 10.

20

TABLE 10
RESULTS OF DTH TESTING WITH REPRESENTATIVE ANTIGENS

	TbH-9 Pos/Total	Tb38-1 Pos/Total	TbRa35 Pos/Total	Cumulative Pos/Total	TbRa11 Pos/Total
PPD negative	0/2	0/2	0/2	0/2	
PPD positive					
healthy	5/9	4/9	4/9	6/9	1/4
prior TB	3/5	2/5	2/5	4/5	3/5
active	3/4	3/4	0/4	4/4	1/4
TOTAL	11/18	9/18	6/18	14/18	5/13

5

EXAMPLE 6

SYNTHESIS OF SYNTHETIC POLYPEPTIDES

10 Polypeptides may be synthesized on a Millipore 9050 peptide synthesizer using Fmoc chemistry with HPTU (O-Benzotriazole-N,N,N',N'-tetramethyluronium hexafluorophosphate) activation. A Gly-Cys-Gly sequence may be attached to the amino terminus of the peptide to provide a method of conjugation or labeling of the peptide. Cleavage of the peptides from the solid support may be carried

15 out using the following cleavage mixture: trifluoroacetic acid:ethanedithiol:thioanisole:water:phenol (40:1:2:2:3). After cleaving for 2 hours, the peptides may be precipitated in cold methyl-t-butyl-ether. The peptide pellets may then be dissolved in water containing 0.1% trifluoroacetic acid (TFA) and lyophilized prior to purification by C18 reverse phase HPLC. A gradient of 0%-60% acetonitrile

20 (containing 0.1% TFA) in water (containing 0.1% TFA) may be used to elute the peptides. Following lyophilization of the pure fractions, the peptides may be characterized using electrospray mass spectrometry and by amino acid analysis.

EXAMPLE 7

PREPARATION AND CHARACTERIZATION OF *M. TUBERCULOSIS* FUSION PROTEINS

5 A fusion protein containing TbRa3, the 38 kD antigen and Tb38-1 was prepared as follows.

Each of the DNA constructs TbRa3, 38 kD and Tb38-1 were modified by PCR in order to facilitate their fusion and the subsequent expression of the fusion protein TbRa3-38 kD-Tb38-1. TbRa3, 38 kD and Tb38-1 DNA was used to perform PCR using the primers PDM-64 and PDM-65 (SEQ ID NO: 146 and 147), PDM-57 and PDM-58 (SEQ ID NO: 148 and 149), and PDM-69 and PDM-60 (SEQ ID NO: 150 and 151), respectively. In each case, the DNA amplification was performed using 10 µl 10X Pfu buffer, 2 µl 10 mM dNTPs, 2 µl each of the PCR primers at 10 µM concentration, 81.5 µl water, 1.5 µl Pfu DNA polymerase (Stratagene, La Jolla, CA) and 1 µl DNA at either 70 ng/µl (for TbRa3) or 50 ng/µl (for 38 kD and Tb38-1). For 15 TbRa3, denaturation at 94°C was performed for 2 min, followed by 40 cycles of 96°C for 15 sec and 72°C for 1 min, and lastly by 72°C for 4 min. For 38 kD, denaturation at 96°C was performed for 2 min, followed by 40 cycles of 96°C for 30 sec, 68°C for 15 sec and 72°C for 3 min, and finally by 72°C for 4 min. For Tb38-1 denaturation at 94°C for 2 min was followed by 10 cycles of 96°C for 15 sec, 68°C for 15 sec and 72°C for 1.5 min, 30 cycles of 96°C for 15 sec, 64°C for 15 sec and 72°C for 1.5, and finally 20 by 72°C for 4 min.

The TbRa3 PCR fragment was digested with NdeI and EcoRI and cloned directly into pT7⁺L2 IL 1 vector using NdeI and EcoRI sites. The 38 kD PCR fragment was digested with Sse8387I, treated with T4 DNA polymerase to make blunt ends and then digested with EcoRI for direct cloning into the pT7⁺L2Ra3-1 vector which was 25 digested with StuI and EcoRI. The 38-1 PCR fragment was digested with Eco47III and EcoRI and directly subcloned into pT7⁺L2Ra3/38kD-17 digested with the same enzymes. The whole fusion was then transferred to pET28b NT LMEIF - 1 using NdeI and EcoRI sites. The fusion construct was confirmed by DNA sequencing.

30 The expression construct was transformed to BLR pLys S *E. coli* (Novagen, Madison, WI) and grown overnight in LB broth with kanamycin (30 µg/ml)

and chloramphenicol (34 µg/ml). This culture (12 ml) was used to inoculate 500 ml 2XYT with the same antibiotics and the culture was induced with IPTG at an OD560 of 0.44 to a final concentration of 1.2 mM. Four hours post-induction, the bacteria were harvested and sonicated in 20 mM Tris (8.0), 100 mM NaCl, 0.1% DOC, 20 µg/ml Leupeptin, 20 mM PMSF followed by centrifugation at 26,000 X g. The resulting pellet was resuspended in 8 M urea, 20 mM Tris (8.0), 100 mM NaCl and bound to Pro-bond nickel resin (Invitrogen, Carlsbad, CA). The column was washed several times with the above buffer then eluted with an imidazole gradient (50 mM, 100 mM, 500 mM imidazole was added to 8 M urea, 20 mM Tris (8.0), 100 mM NaCl). The eluates containing the protein of interest were then dialyzed against 10 mM Tris (8.0).

The DNA and amino acid sequences for the resulting fusion protein (hereinafter referred to as TbRa3-38 kD-Tb38-1) are provided in SEQ ID NO: 152 and 153, respectively.

~~A fusion protein containing the two antigens TbH-9 and Tb38-1 (hereinafter referred to as TbH9-Tb38-1) without a hinge sequence, was prepared using a similar procedure to that described above. The DNA sequence for the TbH9-Tb38-1 fusion protein is provided in SEQ ID NO: 156.~~

The ability of the fusion protein TbH9-Tb38-1 to induce T cell proliferation and IFN-γ production in PBMC preparations was examined using the protocol described above in Example 1. PBMC from three donors were employed: one who had been previously shown to respond to TbH9 but not Tb38-1 (donor 131); one who had been shown to respond to Tb38-1 but not TbH9 (donor 184); and one who had been shown to respond to both antigens (donor 201). The results of these studies (Figs. 5-7, respectively) demonstrate the functional activity of both the antigens in the fusion protein.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for the purpose of illustration, various modifications may be made without deviating from the spirit and scope of the invention.